LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

FUNCTIONAL ALTERATIONS AND EXPRESSION OF AHR RELATED GENES IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF PANCREATIC ADENOCARCINOMA PATIENTS

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2. ABSTRACT

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Pancreatic cancer remains one of the most aggressive cancers with a very poor five-year survival rate (4-5%). Pancreatic adenocarcinoma (PDAC) is responsible for a great majority of all pancreatic cancer cases. The curative intent surgery is possible only in 10-20% of patients and other therapies provide only a modest prolongation of survival. As cancer immunotherapy has a limited efficiency in treating pancreatic cancer, a more personalized multi-modal approach is required to improve the efficiency of pancreatic adenocarcinoma treatment. The aryl-hydrocarbon receptor (AhR) is a ligand-dependent transcription factor, which regulates cell proliferation, chronic inflammation, immunosuppression, cell death inhibition and many other tumorigenic features directly or via its linked genes. Our aim is to investigate the alterations in AhR gene expression in peripheral blood mononuclear cells of 16 II-III stage PDAC patients and 16 healthy volunteers. We also aim to determine the changes of AhR linked genes expression in subgroups of PDAC patients that exhibit similar AhR expression. Our targeted genes ELAVL1, HO1, PD1, PDL1 and IL1, IL4, IL6, IL10, IL12 were investigated under real-time PCR. Additionally, we performed a phagocytic functional analysis. The results of our study demonstrated a significant distinction of high and low AhR expression between the PDAC patients. The high AhR expression group presented with an increased phagocytic activity and higher expression of IL6 and PDL1 genes. Our results suggest that AhR plays a significant role in regulating immune response and tumor cell death in PDAC patients.

3. ACKNOWLEDGEMENTS

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4. CONFLICT OF INTEREST

The author declares that she has no conflicts of interest.

5. RESEARCH ETHICS COMMITTEE APPROVAL

Ethical approval was issued by the Ethics Committee of the Lithuanian University of Health Sciences (Nr. BE-2-62) on the 14th of April 2022. Consent for the use of blood samples specimens for research purposes was obtained from all the patients or their representatives.

6. ABBREVIATIONS

AhR repressor (AHRR)

Aryl hydrocarbon receptor nuclear translocator (ARNT)

Aryl- hydrocarbon receptor (AHR)

Cluster of differentiation 4/8 (CD4/8)

Embryonic lethal vision like protein 1 (ELAV1) or (HuR)

Embryonic lethal vision like protein 1 gene (ELAVL1)

Ethylenediaminetetraacetic acid (EDTA)

Heme-oxygenase 1 (HO1)

Hu antigen R (HuR) ir (ELAV1)

Indoleamine 2,3-dioxygenase (IDO)

Interferon gamma (IFNy)

Interleukin (IL1b/4/6/10/12)

Kynurenine (KYN)

Lipopolysaccharide (LPS)

Major histocompatibility complex (MHC)

Messenger ribonucleic acid (mRNA)

Pancreatic ductal adenocarcinoma (PDAC)

Peripheral blood mononuclear cells (PBMC)

Phosphate buffered saline (PBS)

Polymerase chain reaction (PCR)

Programmed cell death protein 1 and ligand 1 (PD1/PDL1).

Reactive oxygen species (ROS)

Roswell Park Memorial Institute (RPMI) medium

T-cell receptor (TCR)

T-helper cell 1 (Th1)

Tryptophan dioxygenase (TDO)

Tumor microenvironment (TME)

Tumor necrosis factor alfa (TNF-alfa)

Xenobiotic-responsive elements (XRE)

7. INTRODUCTION

Pancreatic cancer remains one of the most aggressive human malignancies with a scarce overall prognosis [1,2]. Pancreatic cancer has limited therapeutic options due to its complexity at genomic, epigenetic, and metabolic levels. Crosstalk at regulatory molecular and cellular levels both complicates the development of new multi-modal therapies and highlights the need for personalized medicine [3].

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor, that modulates a number of AhR-dependent genes, involved in cell proliferation, chronic inflammation, immunosuppression, cell death inhibition and other tumorigenic properties [4]. AhR plays a role not only in tumor development, but also in therapeutic resistance (resistance to chemo- and/or radiotherapy). Such features are critical in developing novel therapies for immunotherapy resistant pancreatic ductal adenocarcinoma (PDAC) - the most diagnosed form of pancreatic cancer [1,3].

Our study involved the real-time polymerase chain reaction of targeted genes in order to investigate the AhR expression between the PDAC patients and AhR effect on other genes expression in peripheral blood mononuclear cells (PBMC) from 16 PDAC patients, who underwent surgery as well as 16 healthy subjects. PBMC (lymphocytes and monocytes) were investigated due to ease of collection, storage, and effectivity in measuring gene expression. PDAC patients' samples were compared to healthy volunteers. Several soluble molecular messengers including IL1b, IL4, IL6, IL10, IL12a, IL12b were targeted because they either belong to the AhR pathways or play a significant role in tumorigenesis [5–8]. Additionally, we investigated the gene expression of an embryonic lethal vision like protein 1 (ELAVL1), a post-transcriptional regulator, that is usually increased in malignancies and is responsible for cytoprotection and inhibition of apoptosis by stabilization of several genes, including heme-oxygenase 1 (HO1) [9]. HO1 is ferroptosis mediator, with a double-sword effect in tumor microenvironment modulation and thus was also included in our study [10]. In order to test an AhR role in cell death and immunotherapy, we included programmed cell death protein 1 and ligand 1 (PD1/PDL1). PD1/PDL1 are immune checkpoint inhibitors, that promote cancer cells survival and immunosuppression [11]. We also performed phagocytic functional analysis in accordance with AhR gene expression, because AhR has been reported to influence phagocytosis in a course of tumorigenesis [8].

As there is limited data on AhR and its linked genes in PBMC of PDAC, we aim to investigate the AhR expression effect on targeted genes PD1, PDL1, ELAV1, HO1, IL1,4,6,10,12 expression. AhR is an established regulator of inflammation magnitude in tumorigenesis, and we expect a more pro-inflammatory and pro-tumorigenic microenvironment within PBMC of patients with high AhR expression. It is likely that high AhR group will present with increased phagocytic activity, as AhR has been linked to phagocytic activity regulating cytokines such as IL4, IL6, IL10. Moreover, considered

that PD1 is a member of Kyn-AhR pathway, we anticipate that PD1 will be upregulated in the AhR high group.

8. AIMS AND OBJECTIVES

Pancreatic cancer, as one of the major causes of human malignancies mortality, has limited therapeutic approaches that provide only a modest prolongation of survival. Pancreatic adenocarcinoma (PDAC) is responsible for majority of pancreatic cancer cases. Hence, there is a need of novel personalized and multimodal treatment strategies development. The aryl- hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that modulates several tumorigenic signaling pathways. Our study investigated AhR and its linked genes in peripheral blood mononuclear cells (PBMC) of 16 PDAC patients (stage II-III) and 16 healthy controls using real-time polymerase chain reaction.

The aim of the study is to investigate how AhR expression affect its targeted genes PD1, PDL1, ELAV1, HO1, IL1,4,6,10,12 expression in PBMC from PDAC patients. Additionally, we performed the phagocytic functional analysis in PBMC in accordance with the AhR gene expression.

The objectives of our study are the following:

1. To assess the AhR expression in pancreatic adenocarcinoma patients;
2. To determine the changes of PD1/PDL1; ELAV1/HO1; IL1,4,6,10,12 expression levels in pancreatic adenocarcinoma patients dependent on the AhR expression;
3. To assess the function of PBMCs by measuring their phagocytic activity in pancreatic adenocarcinoma patients.

9. LITERATURE REVIEW

9.1 PANCREATIC CANCER RELEVANCE AND DEVELOPMENT

Globally, pancreatic cancer is the 7th leading cause of cancer mortality and the 12th most common malignancy [2]. For decades it remains one of the most aggressive human cancers with a very poor survival rate due to difficulties in screening, diagnosis, low response to treatment and high likelihood of a relapse [1,2]. The burden of pancreatic cancer is on an increasing trend and more effective diagnostic and treatment options are in need. The higher incidence and mortality of pancreatic cancer are associated with modifiable risk factors such as smoking, alcohol drinking, reduced physical activity as well as high cholesterol and hypertension [2].

Pancreatic tumors arise from either endocrine or exocrine cells within pancreas, with pancreatic adenocarcinoma (PDAC) forming the great majority of all cases [1]. Tumorigenesis occurs in a step-wise manner, induced by genomic instability, chromosomal rearrangements, accumulative epigenetic changes, followed by precursor dysplasia, loss of tumor suppressor genes and activation of oncogenes [12]. Molecular changes progress into histological alterations with formation of precursor lesions, increasingly higher histological grades and progression to invasive adenocarcinoma [3]. Tumor development leads to changes in the surrounding stroma, by disrupting its normal homeostatic response to injury. Tumor cells develop complex ecosystems, that comprise extracellular matrix, neoplastic cells, infiltrated inflammatory immune cells, and other accessory cells that allow the crosstalk between the cells to ensure further tumor progression [13].

One of the qualities of these highly specialized tumor microenvironments (TME) is chronic inflammation. By evasion of immunity, cancer-associated inflammation contributes to a persistent stimulation of cellular turnover, stem-cell division, and mutagenic effects, anti-apoptotic activity, angiogenesis and eventually cancer dissemination [14]. With tumor progression primary tumoricidal effector response via natural killer cells, CD8+, Th1 Cd4+, cytotoxic macrophages and neutrophils is overbalanced by increased production of pro-tumoral macrophages, regulatory T and B cells, immature dendritic cells and pro-metastatic neutrophils production [13]. This way tumor establishes an immunosuppressive TME by losing expression of tumor antigens and avoiding the recognition by cytotoxic T cells and T cells effector molecules as IL12 induced TNF-alfa or IFNy. In addition, TME continuously recruits immunosuppressive and proinflammatory molecules such as IL4 as well as other tumor-derived chemokines that contribute to this immune-tolerant apparatus. IL4, a pro-inflammatory T-helper 2 cytokine present on both immune cells and tumor itself, is linked to initiation of several tumorigenic activities such as enhanced survival, proliferation, invasion, migration as well as some immune regulatory roles [15]. Overexpression of IL4 in the tumor microenvironment was found to

promote tumorigenesis in both autocrine and paracrine manners and, thus, IL4 is considered as an independent prognostic factor for disease-free survival in PDAC patients after operatively treatment [16]. The complexity of TME system highlights the importance of multimodal therapies aimed at disrupting different features of pancreatic cancer.

9.2 CANCER TREATMENT

Pancreatic cancer treatment consists of surgery, radiotherapy, chemotherapy, or their combination, depending on a disease stage and patient's performance status. Surgical resection is the mainstay treatment including pancreaticoduodenectomy for head or body pancreatic tumors and distal pancreatectomy for tail pancreatic tumors [17]. Although, over the decades the operative mortality has improved astoundingly, operative morbidity after pancreaticoduodenectomy remains dramatically high (40-50%). Nevertheless, as to date, surgical resection is the only curative treatment.

However, only 15 - 20% of pancreatic cancer patients are eligible for surgical resection; 40% of patients are diagnosed at the time of metastatic disease likely due to unspecific symptoms and 30 - 40% have locally advanced unresectable tumors with invasion into adjacent structures such as celiac or superior mesenteric arteries [18]. Therefore, a great majority of pancreatic cancer patients rely on chemotherapy or chemoradiotherapy only, which, unfortunately, result in significantly lower median survival compared to patients with resectable tumors [19]. Intratumor genomic, phenotypic and antigenic heterogeneity highly contribute to therapy failure and pose serious clinical challenges [13]. Despite the fact that immune cells of TME play a key role in development of pancreatic cancer, PDACs are known as immunologically 'cold' or 'non-immunogenic' tumors presenting with low immune recognition [3]. PDAC genomic datasets showed that only a subtype of pancreatic tumors can be targeted by immune modulators [20].

Cancer immunotherapy aims to activate T cell-mediated adaptive immunity by stimulating antigen-presenting cells to recognize cancer as a pathogen and by primed T cells expansion [21]. Development of anti- programmed death receptor/ligand 1 (PDL1/PD1) antibodies (nivolumab, pembrolizumab and atezolizumab, avelumab, respectively), was a breakthrough in cancer monotherapy (Fig 1). Blocking the PDL1/PD1 signaling pathway reactivates immune cells within tumor environment and fights cancer cells. Unfortunately, PDAC lacks the T cell infiltration allowing the inflammatory signaling to activate PDL1 expression [22]. Additionally, immunotherapy presents with drug resistance, the efficacy of immunotherapy is often unsatisfactory in a number of cancers and has to be studied further, especially in developing multi-factorial, personalized combination therapy, that may enhance the anti-tumor effect individually [23]. For instance, there has been a link found between PD1 and an inflammatory mediator IL1b, expressed by T lymphocytes, fibroblasts, epithelial and endothelial cells

[5]. IL1b pro-tumorigenic properties include immunosuppression, neoangiogenesis and enhancement of tumor cell survival. Neutralization of IL1b significantly enhanced the anti-tumor activity of PD-1 in addition to increased CD8+ T cells infiltration, weakened growth of pancreatic neoplasia and led to survival advantage to PDAC-bearing mice [5].

Such and many other ways of targeting a complex nature of PDAC immunosuppression suggest that combination of therapeutic strategies aiming at different TME components, complimentary pathways and feedback responses may increase the efficacy of therapeutic strategies [3]. One of the promising and potent tumor development regulator is reported to be the aryl hydrocarbon receptor (AhR).

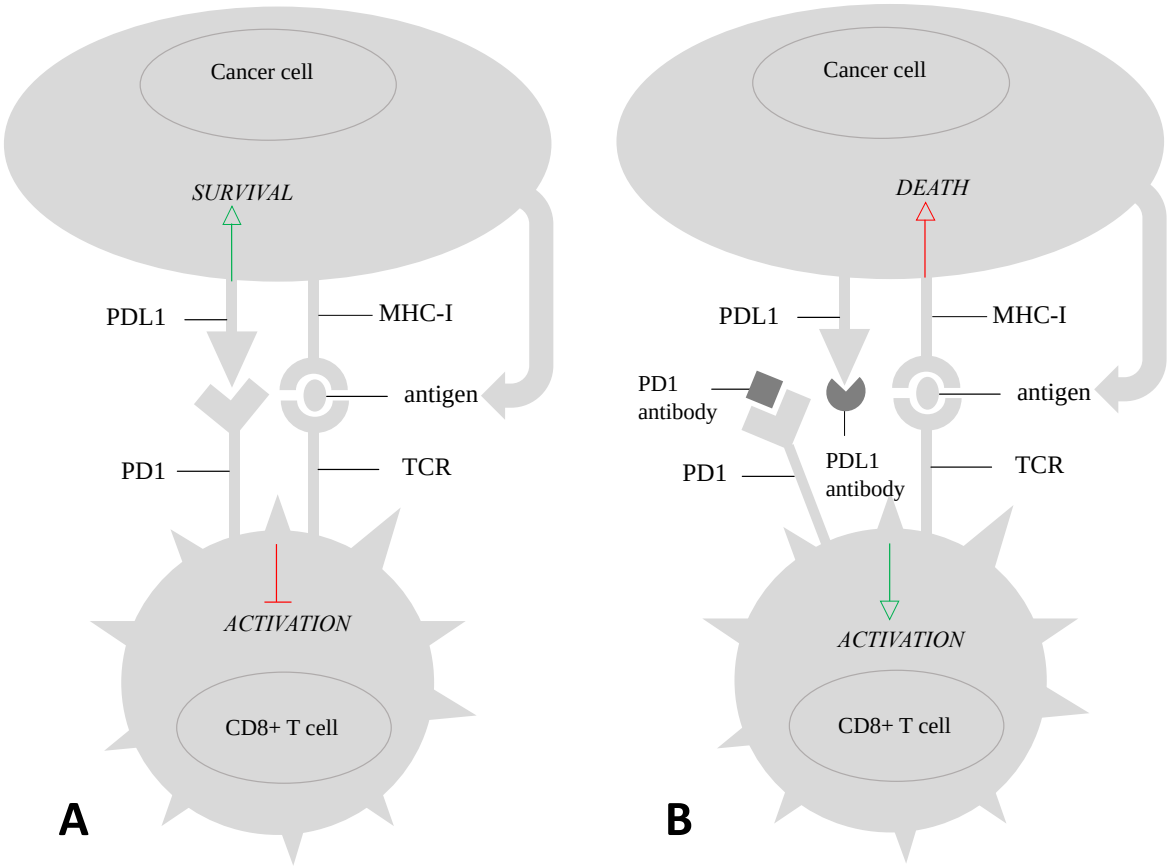

Figure 1: Immune check point inhibition and cancer cells fate. In physiological conditions, the T-cell receptor (TCR) of CD8+ cytotoxic cells binding to tumor-produced antigen as well as the major histocompatibility complex (MHC) protein I on tumor cells enables the recognition of foreign antigen and thus promotes tumor cell lysis by CD8+ T cells. In the tumor microenvironment, the checkpoint protein programmed death cell surface receptor's (PD1) binding to its ligand (PDL1), which is overexpressed on tumor cells, activates the downstream signaling pathway inhibiting T cell activation and promoting cancer cell survival (A). The development of PDL1/PD1 antibodies such as nivolumab, pembrolizumab and atezolizumab, avelumab enables blocking of the binding of PDL1/PD1 and allows CD8+ T cells induced cancer cell death (B). Unfortunately, pancreatic ductal adenocarcinoma cells lack the T cell infiltration as well as PD1 expression as a non-immunogenic tumor and hence monotherapy with immunological checkpoint inhibitors is to date futile.
Adapted from ***Topalian SL, Taube JM, Anders RA, Pardoll DM. Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy. Nature Reviews Cancer. 2016.***

9.3 THE ARYL HYDROCARBON RECEPTOR

The aryl hydrocarbon receptor (AhR) is a cytoplasmic receptor of low tissue specificity, responding to numerous exogenous and endogenous signals. By functioning as a ligand-dependent transcription factor, AhR enables cells to adapt to an ever-changing environment [24]. Different ligands activate different gene clusters in the AhR genomic pathway and subsequently modulate the transcription of AhR-dependent genes [25]. Some of the variety of ligands include xenobiotics, flavonoids as well as kynurenic acid, an endogenous oncometabolite. AhR activity can be also modulated independently of ligand via AhR and aryl hydrocarbon receptor nuclear translocator (ARNT) heterodimerization in cells with high AhR levels induced by shear stress in endothelial cells [4].

9.4 AHR ACTIVATION AND TUMORIGENESIS

Generally, after ligand induced conformational changes, cytosolic AhR is translocated into the nucleus, forming an active heterodimer with the ARNT [25,26]. The newly formed AhR/ARNT complex binds the xenobiotic-responsive elements (XRE) in addition to coregulators in the site of promoters of target genes, regulating their transcription. Besides AhR direct target genes, coregulated AhR genes also play a role in the AhR response [27]. Transient AhR activation is coupled to a negative feedback system via AhR repressor (AHRR) transcription, allowing the degeneration of the activated from of AhR, de novo protein synthesis and the return to normal basal AhR levels [26]. This negative feedback loop prevents deleterious long-term AhR activation. Continuous activation of AhR was found to be damaging as well as a complete AhR loss-of-function [26]. Therefore, it suggests that minimal and well-regulated AhR activity should be beneficial. In its basal state AhR gene functions as a tumor suppressor in tumors of CNS, digestive system, skin and reproductive tract [4,27]. Interestingly, AhR -/- mice have exhibited expression of increased proliferative markers and reduction of other tumor suppressor genes [28]. AhR response to ligand in tumor microenvironment is dependent on a complex crosstalk with signaling components, suggesting that its effect on tumorigenesis is contextual. The effect on cellular functions by AhR depends on a cell type, its timing of the cycle and developmental period. AhR has been demonstrated to both promote or inhibit cell proliferation, which is especially relevant in tumor growth [25].

AhR has been shown to act as a positive or negative regulator of tumor development in several types of cancer and may be a double-edged sword in terms of tumorigenesis. There has not been found any recurrent genetic abnormalities such as amplifications, mutation, or deletions of the AhR gene in cancer [27]. However, the genomic data has revealed elevated AhR mRNA levels in almost 70% different tumor types when compared to healthy tissue. Therefore, it is thought that the level of AhR

tumor inducing, or suppressive properties depend on AhR activity modulation and the tissue it is expressed in.

9.5 AHR INDUCES TOLEROGENIC IMMUNITY

AhR role as an oncogene has been demonstrated in several cancer types as it was found to play a role in all stages of tumorigenesis including initiation, promotion, progression and metastasis [4]. In cutaneous melanoma or glioma cells, sustained AhR activation directly or via induced cytokines resulted in cell plasticity, dedifferentiation, impaired immune system functioning [26]. This demonstrates that AhR is significant not only in the context of tumorigenesis, but also therapy resistance.

To achieve a sustained AhR activation, there should be a continuous source of AhR ligands by tumor and its microenvironment or impaired elimination of activated AhR form. The most significant pathways setting up a sustained AhR activation within tumor microenvironment are thought to be tryptophan dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO) that produce kynurenine (KYN), an AhR ligand [4]. Both IDO and TDO genes are upregulated in several tumors, representing the inflammatory microenvironment as a part of coordinated immunosurveillance evasion [4].

AhR activation by KYN evokes generation of immune-tolerant dendritic cells and regulatory T cells by promoting the production of an immunosuppressive mediator IL10, regulatory dendritic and T cells [21]. Reduced IL10 expression is found in loss of AhR and they both, AhR and IL10, were demonstrated to regulate the inflammatory macrophages [8]. Importantly, IL10 was found to be elevated in PDAC as well as another protumorigenic AhR-linked cytokine IL6 [29]. KYN-AhR pathway gene IL6 is recognized as one of the drivers of PDAC pathogenesis by tumor initiation, progression, resistance to apoptosis and metastatic promotion [30]. Its expression is induced at cancer cells and macrophages and its levels corelates with AhR expression in tumors [7]. The level of IL6 has been reported to be positively corelated with the severity of PDAC including disease stage, distant metastasis, poor overall survival. Moreover, owing to tumor-repopulating cells, KYN-AhR pathway evoke PD1 upregulation in CD8+ T cells, collectively allowing the escape from the adaptive immune system surveillance and debilitate from recognizing and eradicating the tumor cells [31].

AhR also modulates an embryonic lethal vision like protein 1 (ELAV1) (also known as Hu antigen R (HuR)) by HuR nuclear retention [32]. It is an ubiquitously expressed post-transcriptional regulator, mediating the RNA stability of genes by binding AU-rich elements (AREs) and competing with decay factors to promote translation [33]. ELAV1 regulates proteins implicated in carcinogenesis and thus ELAV1 is upregulated in malignancies and thought to lead to an increased cell proliferation, cell survival, angiogenesis and evasion of immune system followed by metastasis [9]. Cytoprotection

and inhibition of apoptosis by ELAV1 are regulated via inducing heme oxygenase (HO1) and some other target mRNA stabilization molecules [34]. Heme oxygenase (HO1) is a phase II metabolizing enzyme, detoxifying xenobiotics in response to oxidative stress, cellular injury and disease in normal physiological conditions [10]. HO1 has cytoprotective effects against cellular stress via mediating ferroptosis, an iron- and lipid peroxidation-dependent cell death. However, in hypoxic environment, which is prominent in pancreatic and other solid tumors, HO1 expression is found to be elevated, leading to delayed ferroptosis. This protects cancer cells from apoptosis and autophagy.

Taken together, ligand-activated transcription factor AhR participates in a complex feedback and feedforward signaling network. This allows AhR to regulate the inflammatory magnitude in tissues and disease manifestation via both adaptive and innate immune system machinery. Considered the major role of immune system within tumorigenesis, an in-depth understanding of AhR and its signaling pathways functioning required for novel treatment strategies development.

10. METHODOLOGY

The study included 32 subjects in total: 16 PDAC patients of the stages II-III and the median age of 69 years old and 16 healthy subjects of median age of 57 years old.

Ethical approval was issued by the Ethics Committee of the Lithuanian University of Health Sciences (Nr. BE-2-62) on the 14th of April 2022.

10.1 HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS ISOLATION BY FICOLL

Collected blood with EDTA was centrifuged and followed by plasma removal. 10 ml samples were diluted with phosphate buffered saline (PBS), suspended and poured onto 15 ml of Ficoll Paque Premium, followed by centrifuging for 35 min in 20 °C. The buffy coat was removed, sampled, diluted in PBS and centrifuged for 10 min in 20 °C. The previous step was repeated several times. The concentration of PBMC was calculated using a Tryptan Blue method. Some of the cells were washed with PBC, centrifuged, suspended, and stored at -20 C °for real-time PCR. Another part of the cells was centrifuged, washed with PBC, and instantly used for function analysis (Fig 2).

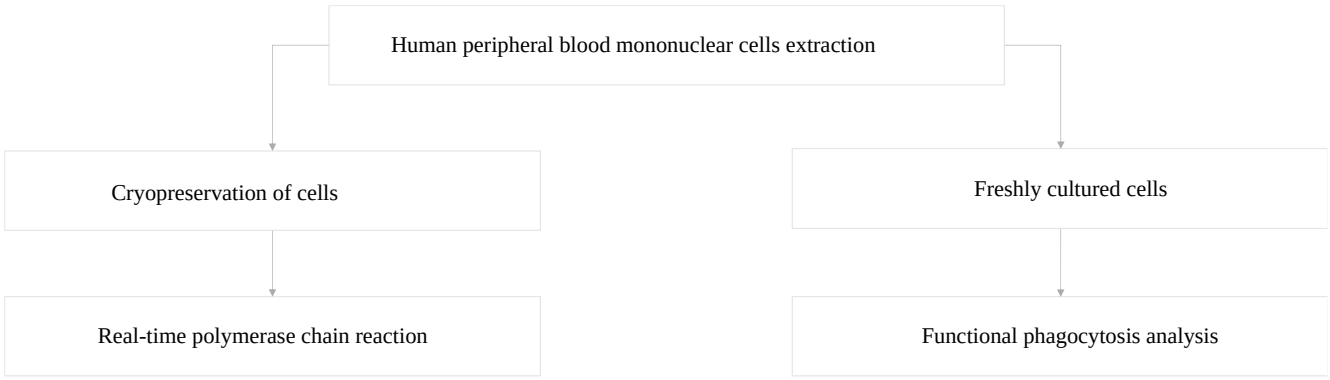

Figure 2: The sequence of experiments. Firstly, human peripheral blood mononuclear cells were isolated and extracted, which followed by division of cells into two groups with aims to perform RT-PCR and functional analysis. The cells for RT-PCR were frozen at -20 °C , while the cells for functional analysis were used instantly.

10.2 RNA EXTRACTION

The RNA isolation of performed with Abbexa's RNA Extraction Kit according to the manufacturer's instructions. Working Binding Buffer was prepared for the PBMC cell number of 5 x 10 ^6 and 1 x 10^7 with 10 ul of beta-Mercaptoethanol for each ml of Binding Buffer.

The sample was prepared by separating PBMC with Ficoll gradient centrifugation (as written before) and frozen. After cells were rethawed, suspension was centrifuged at 12,000 x g for 5 min at 2-8 C °. The supernatant was discarded and Working Binding Buffer was added and vortexed. The solution was homogenized, centrifuged at 12,000 x g for 5 min at room temperature and transferred to a RNase-free tube.

70% ethanol was added to the lysate, vortexed, centrifuged at 12,000 x g for 30 sec and transferred into the spin column. The flow-through was discarded. 500 ul of Clean Buffer was added and centrifuged at 12,000 x g for 30 sec. The flow-through was discarded and the step was repeated. 500 ul of Working Wash Buffer was added and centrifuged at 12,000 x g for 30 sec at room temperature and the flow-through was discarded. The step was repeated. The empty column was centrifuged at maximum speed for 2 min at room temperature and air-dried. The spin column was placed into RNase-free tube and 30- ul of RNase-free Water was added into the spin column matrix and stood for 1 min in room temperature. To elute the RNA, it was centrifuged at 12,000 x g for 2 min. RNA concentration and quality control were investigated using Nano-Drop.

10.3 CDNA REVERSE TRANSCRIPTION

The 2 x RT master mix was prepared. 10ul of 2 x RT master mix and up to 10uL of RNA sample were pipetted (with final mRNA concentration being 1ug/ul). The reverse transcription run was performed in the following steps: at 25 C ° for 10 min, 37 C ° for 120 min, 85 C ° for 5 min and 4 C ° for ∞. The reaction volume was set to 20 uL. The cDNA samples were stored at the temperature of -20 °C.

10.4 REAL-TIME PCR

The following primers were pooled: AHR (bHLHe76) Hs00169233_m1, PD-1 (PDCD1) Hs05043241_s1, PD-L1 (CD274) Hs00204257_m1, IL1B Hs01555410_m1, IL4 Hs00174122_m1, IL-6 Hs00174131_m1, IL10 Hs00961619_m1, IL12A Hs01073450_g1, IL12B Hs99999037 m1, GAPDH Hs02758991_g1, ELAVL1 Hs00171309_m1, HO-1 Hs01110250_m1. The reaction setup included 10 uL of Taq Master mix, 7 uL of H2O, 1 uL of each primer, mixed with 2 uL of cDNA. Each sample was run in duplicated. Real-time PCR was conducted using 7500 Fast Real Time System (Thermo Fisher Scientific) following the manufacturer's thermal cycling protocol for 1 cycle of 2min of denaturation at 50 °C, 1 cycle of 10 min at 95 °C, 40 cycles of 15 sec at 95 °C and 1 min of annealing and extension at 60 °C.

10.5 REAL-TIME PCR STATISTICAL ANALYSIS

Relative quantification with a normalization was used for the result analysis using ΔCT method with a housekeeping gene GAPDH as a references genes. CT of the target gene was normalized for both the patients' and healthy control samples using the formulas: ΔCT(test) = CT(target, test) – CT(ref, test)

and ΔCT(control) = CT(target, control) – CT(ref, control). Secondly, the ΔCT of the test sample was normalized to the ΔCT of the control: ΔΔCT = ΔCT(test) – ΔCT(control). Thirdly, the normalized expression ratio was calculated with the Livak formula 2^(–ΔΔCT) method: 2^(CT(GAPDH) – CT (target)). Medians of each target gene 2^(–ΔΔCT) have been calculated.

To compare samples with high and low AHR expression, the average of AHR from control samples was equated to 1, followed by patients' samples distribution in 2 groups that are of above or below control AHR expression. The significance between the results was calculated using Man Whitney test.

10.6 IMMUNOLOGICAL FUNCTION ANALYSIS OF THE PERIPHERAL BLOOD

The fresh PBMC from patients and healthy samples were repeatedly centrifuged and diluted. The concentration of cells for phagocytic analysis was kept at 1mln/ml cells. RPMI without phenol red was added to the prepared samples. The samples were incubated for 30 min at 37 °C and later spread across dark 96 wells plate with 90 ul of cells mixed with RPMI without phenol red in each well. 10 ul of 0,25 ug/ml lipopolysaccharide (LPS) was added into each well and the plate was incubated. The phagocytic function measurements were taken. 2 hours after the phagocytic function activation, the dark plate was centrifuged, supernatant was removed and 100ul of pHrodo Green Zymosan Bioparticles was added. The measurements in % were taken after an hour. The average of control was equated to 100%.

11. RESULTS

11. 1 DOWNREGULATION OF TARGET GENES IN PDAC

To investigate mRNA expression of target genes, real time PCR was run on the samples of peripheral blood mononuclear (PBMC) from pancreatic ductal adenocarcinoma cells (PDAC) patients and healthy controls. Relative mRNA level expression of target genes was compared (Fig 3). The results revealed that all target genes except of PD1 are downregulated in PDAC in comparison to healthy samples. Expression of IL1b was significantly downregulated in PDAC (p value 0.03) with median of 0.18 (in the range of 0.08 - 0.85) compared to its expression in control with median of 0.9 (in the range of 0.31 - 2.54). HO1 gene expression was also significantly higher in control (p value 0.002) with median of 0.97 (in the range 0.8 of - 1.27) when compared to PDAC with median of 0.58 (in the range of 0.31- 0.67).

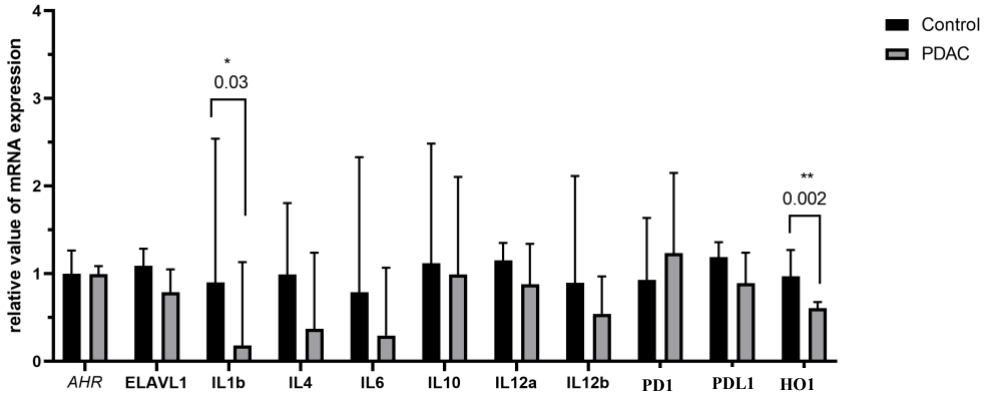

Figure 3: Target genes (AHR, ELAVL1, IL1b, IL4, IL6, IL10, IL12a, IL12b, PD1, PDL1, HO1) expression in peripheral blood mononuclear cells (PBMC) of healthy and pancreatic ductal adenocarcinoma. Each column represents a median target gene expression with interquartile. The gene relative expression was normalized to healthy PBMC. The significant downregulation of IL1b and HO1 is indicated by the p-value. The number of stars represents the weight of significance with ** being the highest.

11.2 LOW AND HIGH AHR EXPRESSION GROUPS IN PDAC

mRNA expression of AhR in PBMC of pancreatic ductal adenocarcinoma samples were divided into two groups of higher and lower AhR expression in comparison to the control AhR expression (Fig 4). Control AhR expression was equated to 1. There was a significant difference detected between the groups (p value <0.0001) with

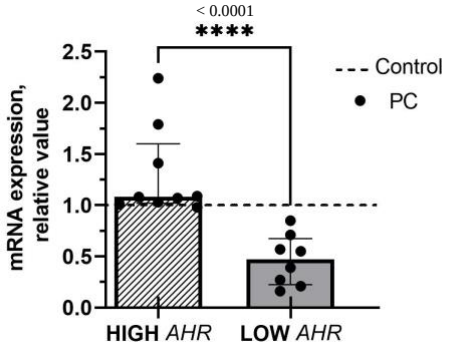

Figure 4: Upregulation and downregulation of AHR expression in peripheral blood mononuclear cells (PBMC) of pancreatic ductal adenocarcinoma
AHR expression in healthy controls was equated to 1 and is identified by an intermittent line. Pancreatic ductal adenocarcinoma samples were grouped into increased and decreased AHR expression in comparison with control AHR. PC indicates AHR expression in individual pancreatic ductal adenocarcinoma cases. The significant downregulation of IL1b and HO1 is indicated by the p-value.

median of 1.08 (in the range of 0.98 - 2.24) in high AhR group and median of 0.47 (in the range of 0.16 - 0.85) in low AhR group.

11.3 INCREASED PHAGOCYTOSIS IN HIGH AHR

Phagocytic activity in peripheral blood mononuclear cells (PBMC) of pancreatic ductal adenocarcinoma was compared in PDAC samples with high and low AhR expression (Fig 5). Control phagocytic activity in healthy samples was equated to 100%. Both groups demonstrated decreased phagocytic activity when compared to healthy control. A significant difference in phagocytic activity between the high AhR and low AhR groups was observed (p value 0.03) with median of 88.45 (in the range of 87.03 - 155.02) in high AhR group and median of 81.16 (in the range of 58.36 - 96.38) in low AhR group.

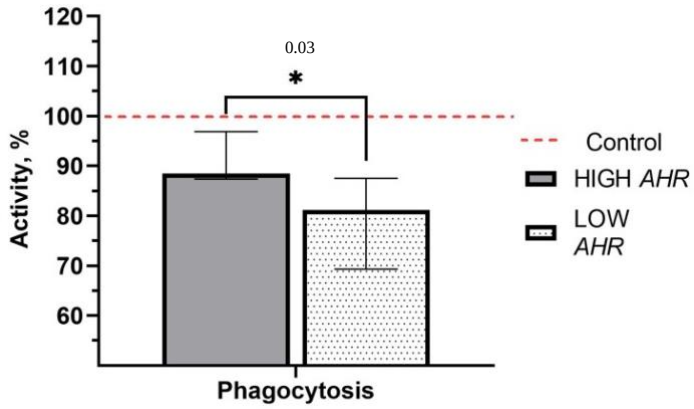

Figure 5: Phagocytosis activity in low and high AHR expression in peripheral blood mononuclear cells (PBMC) of pancreatic ductal adenocarcinoma
Pancreatic ductal adenocarcinoma PBMC samples were grouped by increased and decreased AHR expression. Red intermittent line indicates phagocytosis activity in control samples. Activity of phagocytosis in samples is expressed in %. The significant difference between AHR upregulation and downregulation is indicated by the p-value.

11.4 UPREGULATED GENES EXPRESSION IN HIGH AHR

Target genes AHR, ELAVL1, IL1b, IL4, IL6, IL10, IL12a, IL12b, PD1, PDL1, HO1 expression were compared in high and low AhR expression groups (Fig 6). All target genes in both low and high AhR expression have demonstrated decreased mRNA expression in comparison to control target genes expression. IL6 gene was significantly downregulated in low AhR expression group when compared to high expression group (p value 0.04) with median of 0.35 (in the range of 0.21 - 1.73) in high AhR group and median of 0.23 (in the range of 0.03 - 0.2) in low AhR group. . PDL1 gene was also significantly upregulated in high AhR expression group (p value 0.004) with

median of 1.12 (in the range of 0.84 - 1.26) in high AhR group and median of 0.57 (in the range of 0.3 - 0.63) in low AhR group. .

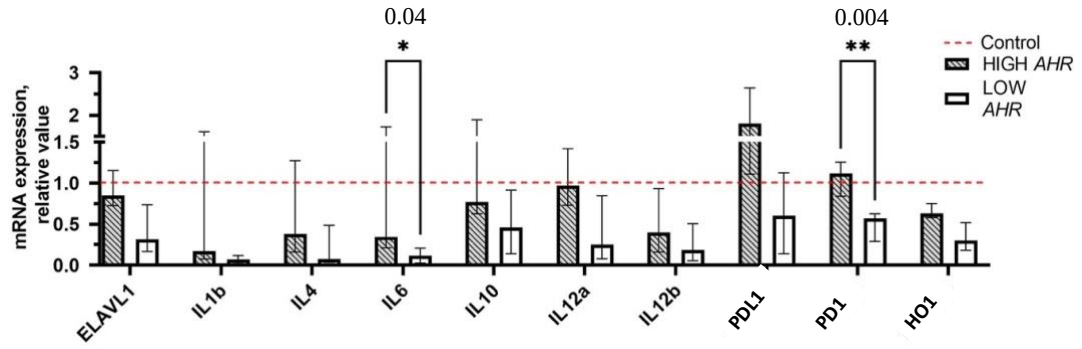

Figure 6: Target genes (AHR, ELAVL1, IL1b, IL4, IL6, IL10, IL12a, IL12b, PDL1, PD1, HO1) expression in in peripheral blood mononuclear cells (PBMC) of pancreatic ductal adenocarcinoma with high and low AHR expression. Each column represents a median target gene expression with interquartile. Striped columns indicate high AHR group and the white ones – low AHR group. Red intermittent line indicates mRNA expression in control samples and is equated to 1. The significant difference between the AHR groups is indicated by the p-value.
The number of stars represents the weight of significance with ** being the highest.

12. DISCUSSION

Increasing incidence and mortality trends in pancreatic cancer require a rapid development of new strategies for earlier diagnosis and a more efficient treatment [2]. Unfortunately, the treatment options of PDAC are limited due to a lack of tumour's immunogenicity and resistance to therapy [3]. AhR is a potent tumorigenesis regulator that affects several signalling pathways and determines the fate of tumour.

It is important to remind the reader that in this study we were measuring the target genes expression, not the level of proteins within PBMC. Although, the gene expression often corelates with its protein level, further research is required to determine the levels of our targeted genes within PBMC.

12.1 IMMUNOLOGICAL MISBALANCE IN PBMC

Tumour development depends on dynamic and reciprocal interactions between cytokines, growth factors, cancerous and non-cancerous cells [35]. To sustain the hallmarks of cancer a great number of modifications are required for cancer to thrive within its microenvironment. Our current study confirmed a misbalance of signalling molecules in PBMC of PDAC. Reduced gene expression of inflammatory mediators such as IL1b, IL4, IL6, IL10 and IL12 suggests a novel composition of signalling molecules within the periphery. IL1b, IL4, are considered to be immunosuppressive, while IL12 is proinflammatory. Decreased expression of these molecules suggest that peripheral immune cells do not necessarily present with pro-tumorigenic and inflammatory genetic profile. The levels of cytokines IL1b, IL4, IL6, IL10 have been reportedly increased in various experiments investigating pancreatic cancer [5,16,29,30]. For this reason, it is critical to run the protein analysis of preceding signalling molecules in our future experiments to compare the gene expression and protein levels. Although PBMC are vital components of the humoral and cell-mediated immunity, these cells reflect the immunological composition in the periphery rather than the epicentre of pancreatic cancer. Hence, our future experiments could additionally investigate the targeted genes expression in other cells involved in pancreatic tumorigenesis such as stromal, fibroblasts, endothelial cells, immune cells of TME and others.

In our study, IL1b presented with a significantly decreased expression in PBMC of PDAC. Generally, IL1b is a tumour-derived inflammatory mediator, which is essential for PDAC microenvironment [5]. Loss of its function is linked to intratumoral infiltration and activation of CD8+ cytotoxic cells. Additionally, IL 1 b was found to be increased in pancreatitis, which is an established pancreatic ductal adenocarcinoma risk factor. Decreased IL1b gene expression in our PDAC samples contradicts the expected increased expression results, suggesting its expression elsewhere than PBMC.

As IL1b is expressed in a wide range of tissues, including digestive tissue macrophages, its expression could be increased in PDAC macrophages [36]. To study such hypothesis, invasive research methods would have to be applied.

Our research also demonstrated a significantly reduced HO1 expression in PBMC of PDAC compared to healthy controls. HO1 functions as a cytoprotective defence activator or ferroptosis-inducing agent depends on ROS production and hypoxia [10]. Hypoxic conditions are prominent in solid tumours' microenvironment and plays a great role in promoting multiple aspects of tumour growth and resistance to cancer chemotherapy as well as radiotherapy [37]. There are findings that in pancreatic cancer cells, HO1 is induced by hypoxia and infection. HO1 inhibition was shown to sensitize the pancreatic cell lines to chemotherapeutic drugs. However, in our study reduced HO1 levels in PBMC of PDAC patients were unexpectedly determined. For the future studies, it is essential to assess the ROS production and oxygen concentration within PBMC in order to investigate a link of HO1 and hypoxia within PBMC. Additionally, HO1 gene expression should be also investigated in other tissues and cells such as tumour-associated macrophages, which would once more require more invasive research methods.

12.2 AHR IS AN IMMUNOREGULATOR VIA IL-6 AND PD1/PDL1

AhR plays a significant role in regulation of inflammation via AhR-dependent signaling pathways. To our surprise, at the first sight when comparing AhR expression in PDAC and healthy controls, there was no significant difference. However, we were able to distinguish individual PDAC samples into two groups with higher and lower gene expression in comparison to AhR expression in healthy control samples. This allowed us to investigate the target genes expression in accordance with high or low AhR expression. Interestingly, PDAC with low AHR expression presented with decreased expression of our target genes. Such results imply that the expression of signaling molecules genes ELAVL1, IL1b, IL4, IL6, IL10, IL12, PDL1, PD1 and HO1 is dependent on AhR expression, and their mRNA expression relative value positively correlates with AhR expression. As reviewed previously, AhR is a potent inflammatory environment regulator within tumorigenesis and our study results complement its importance.

IL6 expression was significantly decreased in the group of low AhR expression. As IL6 is a recognized PDAC pathogenesis driver, together with AhR, it belongs to an important signaling pathway linked to reduced survival in some human tumors [7]. Additionally, the elevation of IL6, a negative prognostic marker in PDAC, is linked to increased disease burden, disease stage, and poor overall survival [30]. IL6 is thought to activate STAT3 signaling pathway. STAT3- regulated genes are linked to PDAC pathogenesis and immune escape strategies. There have been attempts of anti-IL6 targeted

therapy, however no significant improvements were detected [38]. Altogether, the results demonstrate that increased IL6 expression within high AhR group highlights the AhR role as immunosuppression regulator. Future studies should aim to investigate the AhR-IL6 link further in both peripheral immune cells and tumor-associated cells.

As PD1 is mainly expressed on T cells, its engagement with PDL1 suppresses T cell receptor pathway leading to T cell activation inhibition [11]. Thus, PD1/PDL1 is a critical checkpoint inhibitor, responsible for regulation of abnormal cells recognition and elimination. Moreover, Kyn-AhR pathway was reported to evoke PD-1 upregulation in CD8+ T cells, which highlights the influence of AhR on PD1/PDL1 signalling [31]. Unfortunately, unlike breast, colorectal, gastric, non-small cell lung carcinomas, pancreatic cancer does not express abnormally high levels of PDL1 [22]. Our study did show that high AhR expression induced a significantly increased expression of PD1 when compared to low AhR group and that PDL1 gene expression was markedly increased in PDAC when compared to control. This raise hopes that in the future we may possibly regulate the PD1/PDL1 signalling via AhR. To induce a physiological T cell response, we would need to block the PD1/PDL1 binding and thus increase a tumor's response not only to natural immune response, but even possibly to immunotherapy. Since only a handful of PDAC patients respond to immunotherapy aimed at PD1/PDL1 signalling, considered that AhR plays a significant role in PD1/PDL1 gene expression, its normalisation may have an impact on patients' response to immunotherapy. Our following research could investigate the PD1/PDL1 proteins production in the high AhR PDAC group as well as the PD1/PDL1 targeted immunotherapy response in response to increased AhR.

12.3 AHR AND PHAGOCYTOSIS

Separating PDAC patients with low and high AhR expression allowed us to compare the phagocytic activity in both groups and the control. Both AhR expression groups presented with reduced phagocytic activity, which highlights an immunosuppressive environment allowing the tumor growth and development despite AhR expression. However, the difference of phagocytic activity between high and low AhR expression was significant, with the high AhR group showing higher activity than the low AhR group. This suggest that higher AhR expression presents with induced phagocytosis. Such results complement an earlier demonstrated relationship between IL-6 and AhR expression. There have been reports of connection between IL6 and IL4, by which IL6 induced IL4 stimulation produces cancer-promoting M2-like macrophages [16]. It suggests that regulation of AhR expression could alter the immune response via macrophages, a major component of solid cancers possibly via IL6. Additionally, AhR role in macrophage function in pancreatic cancer has been noted earlier: tumor-associated macrophages (TAMs) exhibit high AhR activity and AhR deficient macrophages developed an

inflammatory phenotype [39]. Cell immune checkpoint PD1/PDL1 also plays an important role in regulating phagocytosis in TAMs. Most PD1+ TAMs are M2-like macrophages, promoting the tumor growth efficacy [40]. Taken the results together, high AhR seems to determine a tumorigenic affect, enabling immunosuppression and chronic inflammation by upregulating cytokines such as IL6 and checkpoint inhibitors. Thus, it is critical continue the multi-modal research involving AhR pathway.

12.4 LIMITATIONS OF THE STUDY AND FINAL REMARKS

There are multiple limitations of the study that occurred due to restricted time, a small number of patients pool and technical capabilities. As it was mentioned earlier, it is important to distinguish the gene expression and protein level within cells as they may be uncorrelated. In our study, we aimed to investigate AhR and its linked genes expression in the periphery using the non-invasive methods in PDAC patients.

The results of our study suggest that PDAC patients present with great heterogenicity, which highlights the importance of personalized medicine to improve the therapeutic response once more. The expression of target genes, especially IL6 and PD1, was regulated by AhR expression, which demonstrates that investigating individual expression of AhR in each patient may be significant because it could determine the patient's susceptibility to immunotherapy or severity of the disease (Fig 7). It also raises a question whether AhR stimulation or inhibition could normalize the immune system response to tumor. We also found that increased AhR expression raises phagocytosis. It suggests that regulation of AhR expression could alter the immune response via macrophages, a major component of solid cancers possibly via IL6.

There is a long way ahead in developing an effective treatment of PDAC patients, however AhR signaling pathway may greatly contribute to multi-modal therapies development as it was shown in our study.

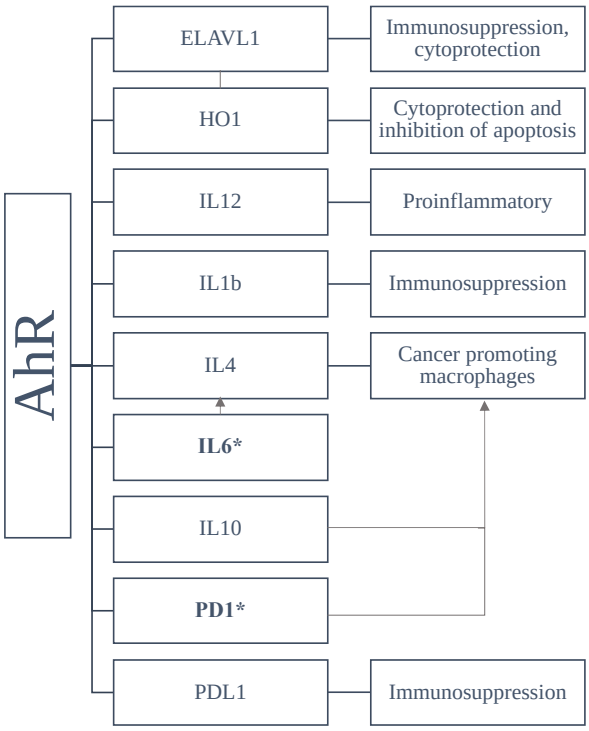

Figure 7: AhR-dependent genes and their role in tumorigenesis. As a potent tumorigenesis and immunity regulator, AhR expression induces increased expression of ELAVL1, HO1, IL12, IL1b, IL4, IL6, IL10, PD1 and PDL1 genes. The forementioned genes have been reported to contribute to tumorigenesis by roles indicated on the right-hand side. ELAVL1 and HO1 link was highligted as cytoprotection and inhibition of apoptosis by ELAV1 are regulated via HO1. Another link between IL6 and IL4 was indicated to demonstrate the IL6-induced IL4 action on phagocytosis. Phagocytic activity has been linked to IL4, IL6, IL10, PD1. The stars represent signalling molecules that we found to be significantly upregulated in the high AhR group.

13. CONLUSIONS

Our study investigated the AhR and its linked-genes expression in peripheral blood mononuclear cells (PBMC) of 16 pancreatic adenocarcinoma (PDAC) patients compared to PBMC of 16 healthy volunteers using real-time PCR. Additionally, we performed a phagocytic function measuring in PBMC of PDAC in accordance with the AhR gene expression. The significance of the result was assessed by p value <0.02.

The conclusions of our study are the following:

1. there is a clear distinguishment between PDAC patients with high and low AhR expression (p value <0.0001) with median of 1.08 (in the range of 0.98 - 2.24) in high AhR group and median of 0.47 (in the range of 0.16 - 0.85) in low AhR group;
2. PDAC with high AhR expression presented with increased expression of PD1 (p value 0.004) with median of 1.12 (in the range of 0.84 - 1.26) in high AhR group and median of 0.57 (in the range of 0.3 - 0.63) in low AhR group;
3. PDAC with high AhR expression presented with increased expression of IL6 (p value 0.04) with median of 0.35 (in the range of 0.21 - 1.73) in high AhR group and median of 0.23 (in the range of 0.03 - 0.2) in low AhR group
4. there is a misbalance of signalling molecules in PBMC of PDAC with significantly reduced IL1b in PDAC (p value 0.03) with median of 0.18 (in the range of 0.08 - 0.85) compared to its expression in control with median of 0.9 (in the range of 0.31 - 2.54);
5. HO1 genes expression was reduced in PDAC (p value 0.002) with median of 0.97 (in the range 0.8 of - 1.27) when compared to PDAC with median of 0.58 (in the range of 0.31- 0.67);
6. PDAC with high AhR expression demonstrated higher phagocytic function activity than the low AhR group (p value 0.03) with median of 88.45 (in the range of 87.04 - 155.02) in high AhR group and median of 81.16 (in the range of 58.36 - 96.38) in low AhR group.

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15. APPENDIX

Dokumentas pasirašytas el. parašu. GINTAUTAS GUMBREVIČIUS
Pasirašiusios šalys: Data: 2022-07-21 21:54:03 GMT+3

KAUNO REGIONINIS BIOMEDICININIŲ TYRIMŲ ETIKOS KOMITETAS
Lietuvos sveikatos mokslų universitetas, A. Mickevičiaus g. 9, LT 44307 Kaunas, tel. (+370) 37 32 68 89;el.paštas: kaunorbtek@lsmuni.lt

LEIDIMAS ATLIKTI BIOMEDICININĮ TYRIMĄ

2022-07-21 Nr. BE-2-62

Biomedicininio tyrimo pavadinimas: „Periferinio kraujo vienbranduolių ląstelių funkcijos sutrikimų dėl galimos potranskripcinės reguliacijos pokyčių kasos vėžio patogenezėje tyrimai“	
Protokolo Nr.:	**1**
Data:	**2022 04 14**
Versija:	**1**
Asmens informavimo forma	**Versija: 2, data: 2022 06 17**
Pagrindinis tyrėjas:	**Prof. Antanas Gulbinas**
Biomedicininio tyrimo vieta:	Lietuvos sveikatos mokslų universiteto ligoninė Kauno
Įstaigos pavadinimas:	klinikos, Virškinimo sistemos tyrimų instituto, Chirurginės gastroenterologijos laboratorija
Adresas:	Eivenių g. 2, LT-50161, Kaunas

Išvada:
Kauno regioninio biomedicininių tyrimų etikos komiteto posėdžio, įvykusio **2022 m. liepos 1 d.** (protokolo Nr. 2022-BE-10-0012) sprendimu pritarta biomedicininio tyrimo vykdymui.

Mokslinio eksperimento vykdytojai įsipareigoja: (1) nedelsiant informuoti Kauno Regioninį biomedicininių Tyrimų Etikos komitetą apie visus nenumatytus atvejus, susijusius su studijos vykdymu, (2) iki sausio 15 dienos – pateikti metinį studijos vykdymo apibendrinimą bei, (3) per mėnesį po studijos užbaigimo, pateikti galutinį pranešimą apie eksperimentą.

Kauno regioninio biomedicininių tyrimų etikos komiteto nariai			
Nr.	Vardas, Pavardė	Veiklos sritis	Dalyvavo posėdyje
1.	Doc. dr. Gintautas Gumbrevičius	Klinikinė farmakologija	Taip
2.	Prof. dr. Kęstutis Petrikonis	Neurologija	Taip
3.	Dr. Saulius Raugelė	Chirurgija	Taip
4.	Doc. dr. Lina Jankauskaitė	Pediatrija	Ne
5.	Prof. dr. Džilda Veličkienė	Endokrinologija	Ne
6.	Doc. dr. Eimantas Peičius	Visuomenės sveikata	Taip
7.	Aušra Degutytė	Visuomenės sveikata	Taip
8.	Dr. Žydrūnė Luneckaitė	Visuomenės sveikata	Taip
9.	Viktorija Bučinskaitė	Teisė	Taip

Kauno regioninis biomedicininių tyrimų etikos komitetas dirba vadovaudamasis etikos principais nustatytais biomedicininių tyrimų Etikos įstatyme, Helsinkio deklaracijoje, vaistų tyrinėjimo Geros klinikinės praktikos taisyklėmis.

Kauno RBTEK pirmininkas Doc. dr. Gintautas Gumbrevičius